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Aspergillus fumigatus during stable state and exacerbations of chronic obstructive pulmonary disease

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Conflicts of Interest

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Author contributions

SM, JA and DD were involved in the recruitment of volunteers and in data collection. AF, VM, JPM and MP were involved in data collection and interpretation. IDP, AJW, CHP were involved in the design of the study, data collection and interpretation. MB and CEB were involved in the study design, volunteer recruitment, data collection, data interpretation, data analysis and had full access to the data and are responsible for the integrity of the data and final decision to submit. All authors contributed to the writing of the manuscript and have approved the final version for submission.

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Short Key Message

Sensitisation to *A. fumigatus* in COPD is related to poor lung function. Culture of filamentous fungi is common but the clinical significance is unclear.

Abbreviation List

Chronic obstructive pulmonary disease	COPD
Allergic bronchopulmonary aspergillosis	ABPA
Aspergillus fumigatus	A. fumigatus
Forced expiratory volume in 1 second	FEV_1
Global initiative for chronic obstructive lung disease	GOLD
C reactive protein	CRP
St Georges Respiratory Questionnaire	SGRQ
Chronic Respiratory Disease Questionnaire	CRQ
Visual analogue scale	VAS
Standard error of the mean	SEM
Interquartile range	IQR
One-way analysis of variance	ANOVA
Cohen Kappa statistic	κ

Abstract (word count 200)

***Background*:** Bacteria are often isolated in stable chronic obstructive pulmonary disease (COPD). Whether fungi are also commonly present and associated with clinical and pathological features of disease is uncertain. We investigated the frequency of filamentous fungal culture and IgE sensitization to *Aspergillus fumigatus* and the relationship to clinical outcomes in COPD subjects.

Methods: COPD subjects were recruited to enter a 1 year observational study. Assessments of lung function, allergen testing and sputum analysis for inflammation, bacteria and fungus were undertaken in COPD subjects and healthy smoking and non-smoking controls.

Results: Filamentous fungi were cultured at baseline in 49% (63/128) of COPD subjects of which 75% (47/63) were *A. fumigatus*. Fungus was cultured in 3/22 controls (2 were *A. fumigatus*). The total sputum cell count and inhaled corticosteroid dosage were significantly increased in COPD patients with a positive filamentous fungal culture at baseline ($p<0.05$). Sensitization to *A. fumigatus* was present in 13% of COPD subjects and was associated with worse lung function (FEV_1 % predicted 39% versus 51%; $p=0.01$), but not related to filamentous fungal culture.

Conclusion: *A. fumigatus* sensitization is related to poor lung function. Positive filamentous fungal culture is a common feature of COPD. The clinical significance of this remains uncertain.

Introduction

Chronic obstructive pulmonary disease (COPD) is associated with significant morbidity and mortality.[1] It is characterized by irreversible airflow obstruction,[2] with underlying emphysema and small airway obliteration which commonly co-exist.[3;4] Airways of patients with COPD are often 'colonized' with potential pathogenic micro-organisms[5] which give rise to increased airway inflammation.[6] Bacteria and viruses have been implicated as the major cause of COPD exacerbations, whereas the potential role of fungal colonization and infection in the pathogenesis of COPD is poorly understood. The commonest fungal genus to cause pulmonary associated fungal infections is *Aspergillus*[7] with a wide spectrum of syndromes including saprophytic invasion, allergic disease and invasive aspergillosis[8] often due to *Aspergillus fumigatus*.[7] Additionally allergic bronchopulmonary aspergillosis (ABPA)[9-12], found commonly in asthma and cystic fibrosis,[13] is increasingly recognized in COPD[14]. Furthermore, sensitization to *A. fumigatus* has been found to be associated with poor lung function in severe asthmatics.[15] Impairment in host defence systems in immunocompetent and immunocompromised patients, including COPD, is thus likely to promote susceptibility to fungal infections. How this impacts on important clinical outcomes in COPD such as disease severity, inflammation and exacerbations is currently unknown.

We hypothesized that the presence of filamentous fungi in the airways of patients with COPD and sensitization to *A. fumigatus* is associated with disease severity, airway inflammation and exacerbations. To study this we carried out a longitudinal study to investigate fungal culture in patients with COPD during stable state and exacerbations.

Methods

Subjects and measurements

Subjects with COPD as per global initiative for chronic obstructive lung disease (GOLD),[2] were recruited as part of a larger biomarker-directed randomized control study.[16] Subjects with a diagnosis of asthma, active pulmonary tuberculosis or any other clinically relevant lung disease were excluded. All COPD subjects had lung function testing including reversibility testing.[17] Sputum was collected for analysis of routine microbiology for potential pathogenic micro-organisms (defined as Haemophilus *influenzae*, Moraxella *catarrhalis*, Streptococcus *pneumoniae*, Staphylococcus *aureus* or Pseudomonas *aeruginosa*)[18] and mycology.[15;19] In brief, approximately 150mg of undiluted sputum was inoculated onto potato dextrose agar plates containing chloramphenicol (16µg/ml), gentamicin (4µg/ml) and fluconazole (5µg/ml). Plates were then incubated at 37ºC and inspected daily for up to 7 days. *A. fumigatus* colonies were identified by colony formation and microscopy. For detection of other filamentous fungi polymerase chain reaction (PCR) sequencing of the large subunit or the internal transcribed spacer region of the nuclear ribosomal operon was used as previously described.[19]

Sputum was also processed to produce cytospins for assessment of sputum total and differential cell counts.[20;21] Venous blood was collected for assessment of peripheral blood differential cell counts and serum C reactive protein (CRP). Health status and symptom scores were measured using the St Georges Respiratory Questionnaire (SGRQ, University of London, UK),[22] the Chronic Respiratory Disease Interviewer-Administered Questionnaire (CRQ, McMaster University, Hamilton, Canada)[23] and the visual analogue scale (VAS) for the domains of cough, breathlessness, sputum production and sputum purulence.[24] Subjects were seen during stable state and exacerbation visits; sample collection was performed prior

to randomization and institution of any therapy. Non-smoking and non-obstructed smoking controls were invited to attend 1 study visit, with lung function testing, sputum induction and demographic data collection. Skin prick testing was used to assess atopy to *dermatophagoides pteronyssinus*, dog, cat, grass pollen and to the fungi *Alternaria alternata, Aspergillus fumigatus, Botrytis cinerea, Cladosporium herbarum* and *Penicillium chrysogenum* (Alk-Abello, Denmark). Total IgE levels; allergen specific IgE antibody levels to cat, dog, timothy grass, *dermatophagoides pteronyssinus, A. fumigatus*; and *A. fumigatus*-IgG levels were measured using the ImmunoCap 250 system (Phadia, UK).

The study was approved by the Leicestershire, Northamptonshire and Rutland Ethics Committee and all subjects gave informed written consent.

Statistical Analysis

Statistical analysis was performed using PRISM version 4 (GraphPad, San Diego, CA) and SPSS version 16 (SPSS, Inc. Chicago, IL). Parametric and non-parametric data is presented as mean (standard error of the mean, SEM) and median (interquartile range, IQR) unless stated. Log transformed data is presented as geometric mean (95% confidence interval). For comparison of unpaired or paired parametric or non-parametric groups, the Student T-test, Paired T-test, Mann-Whitney test and Wilcoxon matched pairs test was used respectively. For comparison of three groups or more for parametric and non-parametric variables the one-way analysis of variance (ANOVA) or Kruskal-Wallis test was used and the χ^2 test for proportions. Repeatability of *A. fumigatus* culture was assessed in patients with two stable visits 3 months apart, using the Cohen Kappa statistic (κ). Logistic regression analysis was used to assess the relationship of the variables of FEV_1% predicted, sputum eosinophil count

(log-transformed), sputum neutrophil count (log-transformed) and exacerbation frequency with the presence of i) *A. fumigatus* only culture and ii) sensitization to *A. fumigatus* using the block entry method. Standard multiple regression analysis was used to assess the relationship of airway inflammation, exacerbation frequency, fungal culture and fungal sensitization with lung function. Logistic regression goodness to fit was performed using the Hosmer-Lemeshow χ_2 test and the R^2 (true and pseudo R^2 for multiple and logistic regression respectively) was used to estimate the variance explained by the model. A p-value of <0.05 was taken as the threshold of significance.

Results

Filamentous fungal culture and baseline demographic data was obtained in 128 patients with COPD (89 men, 39 women). The mean (SEM) % FEV_1 predicted in the COPD subjects was 48 (3). Control data was available in 22 (8 men, 14 women) subjects with a mean (range) age of 58 (41 to 79) years; mean (range) smoking pack year history of 10 (0 to 30) and a mean (SEM) % FEV_1 predicted of 116 (3). The clinical characteristics of the COPD patients and controls are presented in **table 1**. A filamentous fungus was isolated in 49% (63/128) of COPD patients and was predominantly *Aspergillus* and *Penicillium* species (**table E1**). *A. fumigatus* was cultured in 37% (47/128); whilst identification of any *Aspergillus species* was present in 42% (55/128) of patients. Pathogenic bacteria (H. *influenzae*, M. *catarrhalis*, S. *pneumoniae*, S. *aureus* or P. *aeruginosa*) and *A. fumigatus* co-culture was found in 14% (18/128) of patients at baseline stable state. The proportion of control subjects that had positive filamentous fungal cultures was significantly lower compared to COPD subjects (14% versus 49%, mean difference 36%, 95% CI of difference 14 to 48; p=0.002).

Aspergillus fumigatus culture and clinical outcomes

Patients with *A. fumigatus* were on a higher inhaled corticosteroid dose compared to those who were culture negative (1628 vs. 1389 μg beclomethasone dipropionate equivalent, mean difference 239, 95% CI 0 to 477; p=0.050). There were no differences in health status, exacerbation frequency or % FEV_1 predicted in COPD subjects that were *A. fumigatus* culture positive compared to those that were *A. fumigatus* culture negative (**see table 2**). There was a significant difference in total and percentage sputum neutrophil count but not sputum eosinophils in patients with *A. fumigatus* compared to patients with no fungi or other filamentous fungi (**figure 1**). The total sputum neutrophil count was the most independent predictor of A. *fumigatus* culture with a sensitivity and specificity of 54% and 76% respectively (**table 3**).

Atopy and Aspergillus fumigatus

Atopy defined as a positive skin prick testing and/or elevated allergen specific antibodies was found in 34% of COPD patients. Concordance of serum specific IgE antibodies and skin prick testing to the common allergens, showed a good strength of agreement (κ=0.63, 95% confidence interval 0.49 to 0.76). Sensitization to *A. fumigatus* (positive skin prick test and/or elevated *A. fumigatus* IgE antibodies) was present in 13% of patients. Sensitization to *A. fumigatus*, irrespective of corresponding filamentous fungal culture was associated with lower lung function (FEV_1 % predicted 39% vs. 51%; mean difference 11; 95% CI 3 to 20; p=0.01 **figure 2**). There was no difference in parameters of airway inflammation, health status or exacerbation frequency in those that were or were not sensitized to *A. fumigatus*. The FEV_1 % predicted was the best predictor of *A. fumigatus* sensitization (**table 4**).

Aspergillus fumigatus colonization

The repeatability of *A. fumigatus* culture was examined in 70 subjects at two stable visits three months apart. The κ agreement statistic (95% confidence interval) was -0.04 (-0.29 to 0.21). This was determined to be below that observed by chance alone. Of these patients 13% and 39% were either *A. fumigatus* positive or culture negative at both visits respectively. Neutrophil or eosinophil systemic and airway inflammatory measures were not different between patients who cultured *A. fumigatus*, other filamentous fungi and no fungi neither in the repeated visit, nor in patients that either persistently grew filamentous fungi or never grew fungi on 2 visits.

Aspergillus fumigatus during exacerbations

110 exacerbations with filamentous fungal culture from 80 patients were captured. A positive filamentous fungal culture was found in 38% of all exacerbation events; whilst 28% of all exacerbation events were culture positive to *A. fumigatus*. There was no difference in airway inflammation, change in CRQ, FEV_1 or VAS scores between those that cultured *A. fumigatus* at exacerbation compared to those that did not. Corresponding filamentous fungal and bacterial culture data during exacerbations was available in 79 exacerbation events. Co-culture of *A. fumigatus* and pathogenic bacteria was observed in 14% (11/79) of exacerbation events. There were 44 and 35 bacteria and non-bacteria associated exacerbations of COPD. The acquisition of *A. fumigatus* was not different between bacteria associated and non-bacteria associated exacerbations (20% vs. 55%, p=0.07). There was no change from baseline in lung function, health status and symptom scores in bacteria or non-bacteria associated exacerbations and the culture of *A. fumigatus* at the baseline and exacerbation visit **(see supplement table E2 and table E3)**.

Discussion

Here we report that in subjects with COPD, sensitization to *A. fumigatus* was associated with poor lung function. Positive culture of filamentous fungi especially that of *A. fumigatus* was commonly found in the sputum of patients with COPD and was increased compared to controls. However, repeatability of *A. fumigatus* culture in stable state was poor and the prevalence did not change at exacerbations. The clinical significance of a positive filamentous fungal culture in COPD therefore remains uncertain.

In our study, hypersensitivity to *A. fumigatus* was detected in 13% of the COPD subjects and was associated with reduced lung function independent of *A. fumigatus* sputum culture. This is consistent with previously observed findings in patients with severe asthma using the same fungal culture technique.[15] Whether sensitization to *A. fumigatus* contributes to the cause of airflow obstruction or is a consequence of a damaged and remodelled airway and thus more likely in subjects with severe COPD is uncertain. In severe asthma the relationship between sensitization and filamentous fungal culture suggests that persistent colonization may promote sensitization. Although we were unable to replicate the finding of colonization in our study, it remains plausible that sensitization reflects increased filamentous fungi exposure over time.

Sensitization to *A fumigatus* is a feature of ABPA. Although we did not undertake computed tomography in this study, none of our subjects fulfilled Greenberger's criteria for ABPA.[11;12] The incomplete fulfilment of this ABPA criteria has also been shown in previous COPD studies.[14] In asthma associated with ABPA and in severe asthma with fungal sensitization (SAFS), anti-fungal therapy has demonstrated clinical benefit although the magnitude of this effect is small.[25-27] Whether anti-fungal therapy may be effective in reducing exacerbations

and disease progression in COPD patients with filamentous fungal sensitization remains to be tested.

Colonization of the airways, defined as 'the presence of potentially pathogenic organisms without an associated inflammatory response' is common in patients with COPD, occurring in up to 30%.[5] Using a specialist fungal culture technique, we have shown that the presence of filamentous mould (mainly *A. fumigatus*) was found in almost half of subjects with COPD; and that neutrophilic airway inflammation was the best predictor of *A. fumigatus* sputum culture. The presence of *A. fumigatus* or any filamentous fungal culture was also related to higher doses of inhaled corticosteroids. Immunosuppression from corticosteroid treatment has been associated with invasive aspergillosis and studies have determined that corticosteroid therapy was an independent predictor of invasive aspergillosis in COPD patients in intensive care.[28;29] Current COPD guidelines advocate the use of inhaled corticosteroids in patients with an FEV_1 <50% and recurrent exacerbations to reduce the risk of future exacerbations.[2] At present it remains unclear whether there is an inhaled corticosteroid dose-response predisposition to *A. fumigatus* colonization in a susceptible cohort or an associated increase in risk of invasive aspergillosis and requires further studies.

Colonization of *Aspergillus* in immunocompetent individuals has been defined as one that does not fulfil the criteria for definite or probable invasive aspergillosis.[30;31] However this definition has focussed on filamentous fungal detection from respiratory samples collected on a single episode only. There is a paucity of data in the literature examining repeated *A. fumigatus* culture in COPD subjects at stable state and even less investigating *A. fumigatus* during exacerbations. In this study we could not determine repeatability of *A. fumigatus* culture in subsequent stable visits. However our study highlights the importance of

examining relationships of pathogen and airway inflammation expression in longitudinal studies; and we have aimed to explore the classification of filamentous fungal colonization in the COPD airway and related this to important clinical outcomes. Although we have been unable to show that there is persistence of *A. fumigatus* culture in a cohort of patients with repeated visits, emerging knowledge regarding the complex microbiota of the airways in COPD [32] would suggest that the role of filamentous fungi and in particular *A. fumigatus* needs to be further explored in the pathogenesis of COPD. Our study is strengthened by examining filamentous fungal culture in the airways of non COPD controls; and showed that despite similar atopy rates there was significantly reduced detection of fungal and *A. fumigatus* culture. Although the control group was not aged or smoking matched, the detection of filamentous fungus in the sputum was significantly lower and independent of atopy status. A final concern is whether we have inadvertently included patients with asthma and classed these patients as COPD. However, to reduce this bias a strict exclusion criteria for screening was a current or previous history of asthma, whether self-reported or physician diagnosed, and used current spirometric diagnostic criteria for COPD as per GOLD guidelines[2]. Therefore whether our finding of *A. fumigatus* sensitisation and worsened lung function is related to disease or prolonged exposure need to be further evaluated in larger studies.

To our knowledge, this is the first study to examine filamentous fungal and *A. fumigatus* culture rates during exacerbations of COPD. In contrast to our hypothesis, we detected that filamentous fungal culture rates and in particular *A. fumigatus* was not increased during exacerbations of COPD. Studies have shown that bacterial and fungal interactions include promotion of survival and virulence of fungus, or inhibition of filament formation.[33] Furthermore, the development of bacterial and fungal bio-films may provide a defence host

environment which has resistance to antibiotic properties.[33] In this study we found that *A. fumigatus* culture was not affected by the presence or absence of concomitant bacterial culture and whether interactions between filamentous fungal and bacterial infection during exacerbations are important remains to be fully determined.

In conclusion, we have shown that *A. fumigatus* in commonly found in the sputum of patients with COPD and that this is irrespective of disease severity. We have also shown that IgE sensitization to *A. fumigatus* in COPD subjects is associated with lower lung function and that the detection of *A. fumigatus* by sputum culture is increased compared to controls but was unrelated to exacerbations. However, the clinical significance of fungi in COPD and the response to anti-fungal therapy remains to be determined and further studies are required.

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Table 1; Clinical characteristics of COPD and control subjects

	COPD subjects N=128	Controls N=22
Male, n (%)	89 (70)	8 (36)
Age*	69 (47 to 86)	58 (41 to 79)
Pack years smoked*	54 (10 to 207)	10 (0 to 30)
Atopy, %	34	35
FEV_1 % predicted	48 (3)	116 (3)
Filamentous fungal culture positive, % (n)	49 (63)	14 (3)
Aspergillus fumigatus culture, % (n)	37 (47)	9 (2)

Data presented as mean (standard error of the mean) unless stated. * mean (range)

Table 2; Clinical characteristics of COPD subjects according to filamentous fungal status

	No fungus culture N=65	A *fumigatus* culture N=47	Other filamentous fungus culture N=16	p-value
Male, n (%)	41 (63)	35 (75)	13 (81)	0.24
Age*	68 (47 to 87)	72 (53 to 86)	68 (51 to 82)	0.10
Current smokers, n (%)	**31 (48)**	**11 (23)**	**6 (37)**	**0.03**
Ex-smokers, n (%)	33 (51)	34 (72)	10 (63)	0.07
Pack years smoked*	56 (10 to 207)	49 (10 to 130)	57 (12 to 138)	0.56
Exacerbations in previous yr	3 (1 to 12)	3 (1 to 8)	3 (1 to 10)	0.30
GOLD I, n (%)	3 (5)	2 (4)	1 (6)	0.95
GOLD II, n (%)	27 (42)	16 (34)	3 (18)	0.22
GOLD III, n (%)	19 (28)	16 (34)	6 (38)	0.76
GOLD IV, n (%)	16 (25)	13 (28)	6 (38)	0.58
Inhaled corticosteroid dose¥, µg	**1389 (86)**	**1628 (84)**	**1754 (130)**	**0.05**
Atopy, % (95% CI)	34 (22 to 50)	55 (38 to 70)	14 (1 to 53)	0.07
FEV_1/FVC, %†	49 (1)	49 (2)	46 (4)	0.55
FEV_1 % predicted†	51 (2)	48 (2)	44 (5)	0.38
Peripheral leukocytes, $x10^9$ cells/L¶	8.7 (8.1 to 9.3)	7.8 (7.3 to 8.4)	7.9 (6.8 to 9.2)	0.12
Peripheral blood eosinophils, %¶	2.4 (2.1 to 2.9)	2.5 (2.1 to 3.0)	2.5 (1.7 to 3.8)	0.56
Sputum total cell count, $x10^6$ cells/g¶	**2.1 (1.5 to 3.1)**	**4.1 (2.9 to 5.9)**	**4.1 (2.8 to 5.9)**	**0.03**
Sputum neutrophils, %	**66 (3)**	**78 (3)**	**78 (6)**	**0.03**
Sputum eosinophils, %¶	1.1 (0.6 to 1.2)	0.8 (0.6 to 1.2)	1.7 (0.7 to 4.2)	0.19
Total IgE, kU/L‖	39.4 (156.7)	57.7 (165.5)	46.9 (151.4)	0.64
CRP, mg/L‖	3 (7)	3 (8)	5 (9)	0.65
A. fumigatus specific IgE>0.35, %‡	13	15	0	0.26
A. fumigatus IgG>40, %‡	18	28	15	0.26
SGRQ total, units	56 (2)	53 (2)	47 (4)	0.14
CRQ total, units	3.8 (0.1)	4.2 (0.2)	4.4 (0.2)	0.08
VAS total, mm	167 (10)	162 (13)	157 (11)	0.90

Data presented as mean (standard error of the mean) unless stated. * mean (range); ¥beclomethasone dipropionate equivalent; † post bronchodilator; ¶ geometric mean (95% confidence interval); ‖ median (interquartile range); ‡ Proportion IgE >0.35 or IgG >40. FEV_1 Forced expiratory volume in 1 second; FVC Forced vital capacity; SGRQ St Georges Respiratory Questionnaire, scores ranging from 0 to 100 with a higher score indicating worse health status (Total score made up of Impact, Symptoms and Activity domains); CRQ Chronic Respiratory Health Questionnaire, scores range from 1 to 7 with a higher score representing better health quality (Total score made up of Emotion, Dyspnoea, Fatigue and Mastery domains); VAS Visual Analogue Scale, performed on 100mm line from 'no symptoms' to 'worst symptoms', higher scores represent worse symptoms (Total score calculated as addition of cough, dyspnoea, sputum volume production and purulence domains).

Table 3; Predictors of *Aspergillus fumigatus* sputum culture in COPD subjects at stable state

	Odds Ratio	95% confidence interval	p-value
Exacerbation frequency	0.84	0.69 to 1.03	0.09
% FEV_1 predicted	0.97	0.96 to 1.01	0.32
Sputum eosinophils, %	0.53	0.24 to 1.20	0.13
Total sputum neutrophil count	**1.97**	**1.05 to 3.69**	**0.03**

Table 4; Predictors of *Aspergillus fumigatus* sensitization in COPD subjects at stable state

	Odds Ratio	95% confidence interval	p-value
Exacerbation frequency	1.17	0.89 to 1.55	0.27
% FEV_1 predicted	**0.95**	**0.91 to 0.99**	**0.02**
Sputum eosinophils, %	1.04	0.34 to 3.23	0.94
Total sputum neutrophil count	2.12	0.78 to 5.81	0.14
Aspergillus fumigatus culture	1.23	0.34 to 4.49	0.76

Figure Legends

Figure 1

Percentage and total sputum neutrophil counts (a and b) and percentage sputum eosinophils (c) in patients with *Aspergillus fumigatus* culture (Af +), other filamentous fungi and no fungi cultured at study entry. Horizontal bar set at mean, error bars set at standard error of the mean.

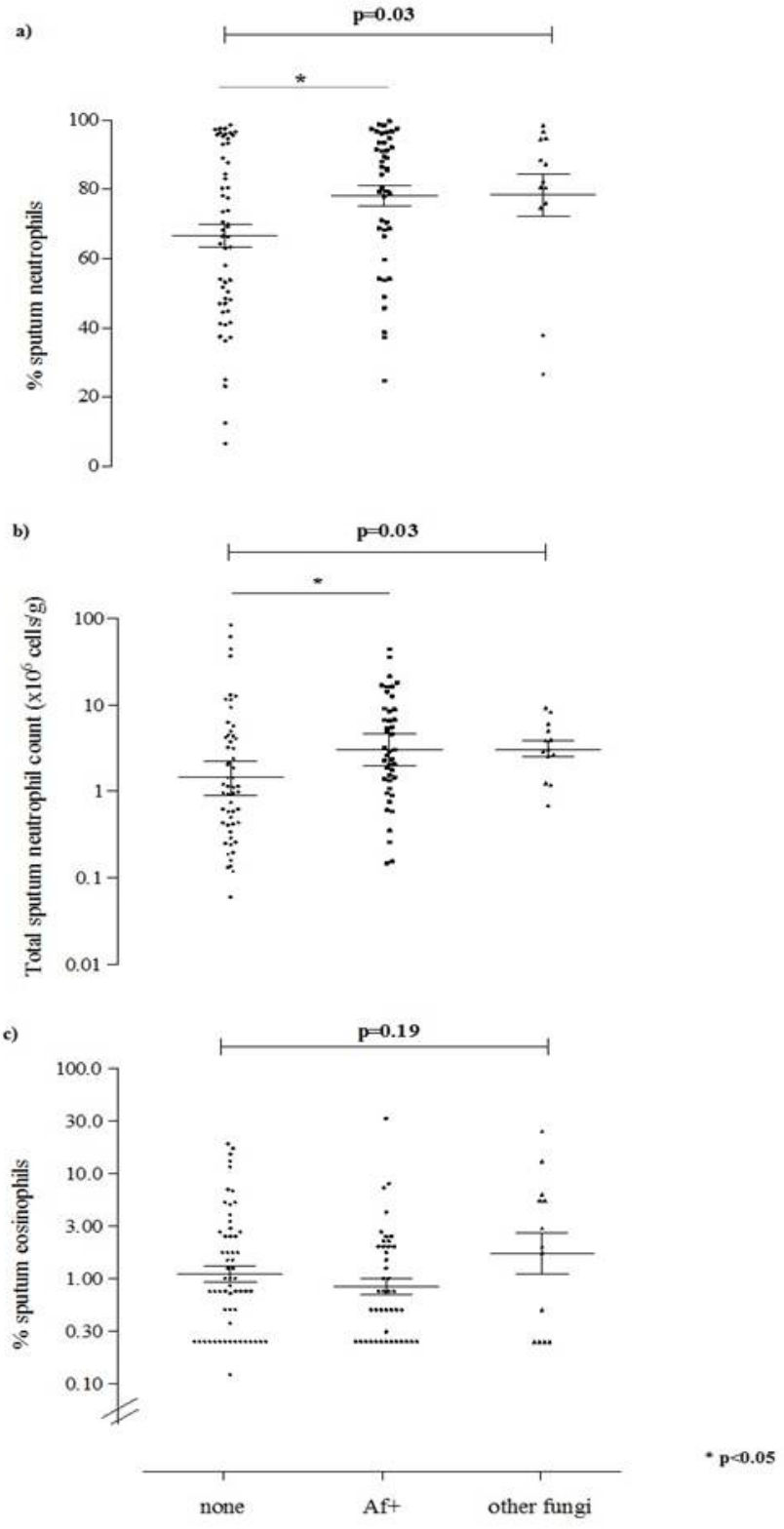
a)
p=0.03
*
% sputum neutrophils
100
80
60
40
20
0
b)
p=0.03
*
Total sputum neutrophil count (x10^6 cells/g)
100
10
1
0.1
0.01
c)
p=0.19
% sputum eosinophils
100.0
30.0
10.0
3.00
1.00
0.30
0.10
* p<0.05
none
Af+
other fungi

Figure 2

FEV_1 % predicted categorised according to *Aspergillus fumigatus* (Af) sensitization. Horizontal bar set at mean, error bars set at standard error of the mean.